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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07D 498/18, C12P 17/18, A61K 31/435 // (C07D 498/18, 311:00, 273:00, 221:00) (C12P 17/18, C12R 1:465)

A1

(11) International Publication Number: WO 94/18207

(43) International Publication Date: 18 August 1994 (18.08.94)

(21) International Application Number: PCT/EP94/00284

(22) International Filing Date: 1 February 1994 (01.02.94)

(30) Priority Data:
9302016.2 2 February 1993 (02.02.93) GB

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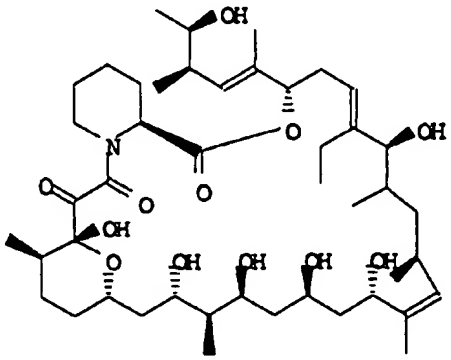
(81) Designated States: AU, BB, BG, BR, BY, CA, CN, CZ, FI, HU, JP, KP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: RAPAMYCIN-LIKE MACROLIDE AND A NEW STRAIN OF STREPTOMYCES WHICH PRODUCES IT



(I)

(57) Abstract

A macrolide of formula (I) that has FK506 and rapamycin antagonistic properties. The macrolide is a metabolite of a newly isolated *Streptomyces* strain, *Streptomyces* sp. A 91-261 402.

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RAPAMYCIN-LIKE MARCROLIDE AND A NEW STRAIN OF STREPTOMYCES WHICH PRODUCES IT

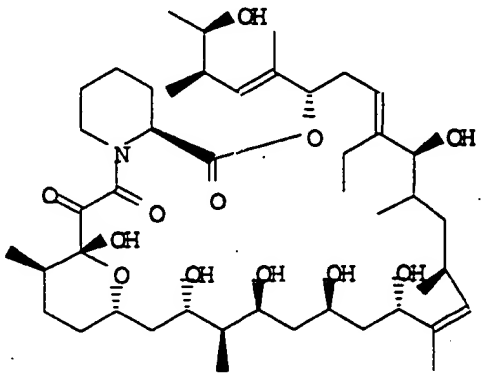
This invention relates to a rapamycin-like macrolide which is an antagonist of FK506 and rapamycin, and to a newly isolated strain of Streptomyces which produces the macrolide.

Rapamycin (sirolimus) is a macrolide antibiotic that is produced by Streptomyces hygroscopicus. The structure of Rapamycin is given in McAlpine et al; 1991; J. Antibiotics, **44**, 688 and Schreiber et al; 1991; J. Am. Chem. Soc., **113**, 7433. Rapamycin is an extremely potent immunosuppressant and also has anti-tumour and anti-fungal activity. However the utility of rapamycin as a pharmaceutical is restricted by its low and variable bioavailability and its high toxicity.

FK506 (tacrolimus) is a macrolide antibiotic that is produced by Streptomyces tsukubaensis No 9993. The structure of FK506 is given in Tanaka et al; 1987; J. Am. Chem. Soc., **109**, 5031. FK506 also is a potent immunosuppressant. However FK506 is also toxic; particularly to the nervous system.

FK506 and rapamycin have somewhat different mechanisms of inducing immunosuppression; however, both bind strongly to common intracellular binding proteins (macrophilins). It is believed that this binding is a prerequisite for their immunosuppressive activity and the immunosuppressive activity of their numerous immunosuppressive derivatives.

This invention provides a novel macrolide which is not immunosuppressive, but which binds strongly to macrophilins. This novel macrolide is useful as a pharmaceutical, e.g., as an antidote to macrophilin binding immunosuppressants of the FK506 or rapamycin type, as a steroid potentiator, and as a Mip inhibitor, as well as in diagnostic and screening assays. The novel macrolide is of formula I:



I

The macrolide of formula I binds to macrophilin with an affinity comparable to that of rapamycin and of FK506.

The macrophilin binding activity of the macrolide of formula I can be shown using the macrophilin binding assay (MBA), which measures the ability of the macrolide of the invention of compete with a macrophilin binding immunosuppressant for binding to a macrophilin. FK506, rapamycin, and immunosuppressive derivatives of these drugs are known to bind in vivo to macrophilin-12 (also known as FK506 binding protein or FKBP-12), so the ability of the macrolide to compete with FK506 or rapamycin for binding to macrophilin-12 can be measured. In this assay, FK506 is coupled to bovine serum albumin (BSA) and is then used to coat microtiter wells. Biotinylated recombinant human macrophilin-12 (biot-MAP) is allowed to bind in the presence or absence of the macrolide of formula I to the immobilized FK506. After washing (to remove macrophilin-12 which is not specifically bound to the immobilized FK506), the amount of bound biot-MAP is assessed by incubation with a streptavidin-alkaline phosphatase conjugate, followed by washing and subsequent addition of p-nitrophenyl phosphate as a substrate. The read-out is the OD at 405nm. Any binding of the macrolide of formula I to the biot-MAP results in a decrease in the amount of biot-MAP that is bound to the FK506 and thus in a decrease in the OD 405. Repeating the test at various concentrations of the macrolide allows the determination of the concentration resulting in 50% inhibition of the biot-MAP binding to the immobilized FK506 (IC50). The inhibitory capacity of the macrolide is then compared to the IC50 of free FK506 as a standard and expressed as a relative IC50 (i.e., IC50-macrolide/IC50-free FK506). A higher relative IC50 corresponds to a lower binding affinity and hence indicates a less powerful antagonist.

The macrolide of formula I is not immunosuppressive and is a FK506 antagonist and can reverse the immunosuppressant effects of FK506. This can be demonstrated using a functional assay of inhibition of IL-2 gene transcription, such as an IL-2 reporter gene assay. FK-506 is known to inhibit the induction of IL-2 transcription in PHA/PMA stimulated JURKAT lymphoma cells and therefore

the ability of the macrolide to block the FK506-mediated inhibiton of IL-2 transcription in an IL-2 reporter gene assay may be used to demonstrate antagonism.

A suitable IL-2 reporter gene assay is that described by Baumann et al. (Transplantation Proceedings (1992), 24:43). This assay uses JURKAT cells which have been stably transfected with a DNA construct comprising the E. coli β galactosidase gene under the control of the human IL-2 gene promoter which is activated by signals which induce IL-2 gene transcription. PHA/PMA stimulation of the transfected cells results in β-galactosidase expression which is measured using a fluorogenic substrate (4-methyl-umbelliferyl-β-galactoside), and reflects activation of IL-2 expression. Cultures are treated with various concentrations of FK506 either alone or in the presence of fixed concentrations of the macrolide and β-galactosidase activity is assessed at 16 hours by addition of the fluorogenic substrate. FK506 inhibits IL-2 and β-galactosidase expression and this inhibition is blocked in the presence of the macrolide.

Similarly the macrolide of formula I is a rapamycin antagonist and can reverse the immunosuppressant effects of rapamycin. This can be demonstrated using an IL-6-dependent cell proliferation assay. Rapamycin is known to inhibit the IL-6 induced proliferation of the IL-6-dependent B cell hybridoma cell line B 13-29, and therefore the ability of the macrolide to block the rapamycin-mediated inhibition of the proliferation of this cell line may be used to demonstrate antagonism.

In this assay, B 13-29 cells are treated with various doses of rapamycin in the presence of fixed concentrations of the macrolide. IL-6-induced cell proliferation (at 0.03 ng IL6/ml) is then measured by ^{3}H-thymidine incorporation at 72 hours. The macrolide blocks the rapamycin-mediated inhibition of cell proliferation in a dose dependent manner, having an antagonistic activity comparable to that of FK506.

Since the macrolide of formula I binds strongly to macrophilin and is a FK506 antagonist and a rapamycin antagonist, it is useful in the treatment of overdoses of macrophilin-binding immunosuppressants, such as FK506 and rapamycin.

The macrophilin binding activity of the compound of the invention also makes it useful in enhancing or potentiating the action of corticosteroids. Combined

treatment with the compound of the invention and a corticosteroid, such as dexamethasone, results in greatly enhanced steroidal activity. For instance, it was found that the compound of formula I exhibits a synergistic effect in combination with glucocorticosteroids, e.g. with dexamethasone, in allergic contact dermatitis in mice. This activity can be shown in the following manner.

Groups of 8 female, 5 week old NMRI mice are sensitized with 10µl of 2% oxazolone applied to the shaved ventral abdomen on day 1. Oxazolone is dissolved in acetone. On day 8 the second exposure (challenge) is performed by the application of 10µl of 2% oxazolone to the inner aspect of the right pinnae of test and control animals. Topical application of the test compounds (dissolved in ethanol) is performed once (30 minutes after the challenge). The efficacy is determined on day 9 by determination of the individual differences of both pinnal weights in test and control animals. The results are summarized in the following table.

Compound of formula I	dexamethasone	inhibition
-	0.01%	67%
-	0.004%	32%
1.2%	-	24%
0.13%	-	28%
0.01%	-	20%
1.2%	0.004%	49%
0.13%	0.004%	59%
0.01%	0.004%	37%

inhibition = pinnal weight vs control

In view of these results the combination of the compound of the invention and a glucocorticosteroid, e.g. dexamethasone, is indicated for use in the topical

treatment of inflammatory/hyperproliferative skin diseases.

For these indications the indicated daily dosage is of course dependent on the mode of administration and the type of treatment. Satisfactory results are obtained in larger mammals with a dosage topically administered of about 0.05-1% of glucocorticosteroid and of about 0.01%-2% of the compound of the invention.

The invention further includes a method of treatment, curative or supportive, of conditions as described above comprising administering to a subject in need of such treatment a therapeutically effective amount of the combination. It further comprises the combination for use in the above indications, expecially for use as an antiallergic agent.

Additionally, the macrolide of the invention binds to and blocks a variety of Mip (macrophage infectivity potentiator) and Mip-like factors, which are structurally similar to macrophilin. Mip and Mip-like factors are virulence factors produced by a wide variety of pathogens, including those of the genera Chlamidia, e.g., Chlamidia trachomatis; Neisseria, e.g. Neisseria meningitidis; and Legionella, e.g. Legionella pneumophilia; and also by the obligately parasitic members of the order Rickettsiales. These factors play a critical role in the establishment of intracellular infection. The efficacy of the macrolide of the invention in reducing the infectivity of pathogens which produce Mip or Mip-like factors can be shown by comparing infectivity of the pathogens in cells culture in the presence and absence of the macrolides, e.g., using the methods described in Lundemose, et a., *Mol. Microbiol.* (1993) 7: 777. The compound of the invention has a marked advantage over FK506, rapamycin and their immunosuppressant derivatives for use in this indication for the reason that it is not immunosuppressive, thus it does not compromise the body's natural immune defenses against the pathogens.

Accordingly this invention also provides a macrolide of the formula I, for use as a pharmaceutical; for example as an antidote for overdoses of a macrophilin binding immunosuppressant, e.g., FK506 or rapamycin; or as a steroid potentiator; or as an anti-infective agent.

The invention also provides a pharmaceutical composition comprising a macrolide of formula I together with a pharmaceutically acceptable diluent or carrier. Preferably the composition is useful for treating the effects of an overdose

of a macrophilin binding immunosuppressant, e.g., FK506 or rapamycin; or as a steroid potentiator (alone or in combination with one or mor corticosteroids); or as an anti-infective agent (alone or in combination with other anti-infective agents).

The invention further provides the use of a macrolide of formula I in the manufacture of a medicament to be used as an antidote for overdoses of a macrophilin binding immunosuppressant, e.g., FK506 or rapamycin; or as a steroid potentiator, e.g., as an anti-inflammatory agent, or for one of the other uses kown for corticosteroids; or as an anti-infective agent, e.g., in the prophylaxis and treatment of infections or infectious diseases caused by organisms producing Mip or Mip-like factors, including organisms of the genera Chlamidia, e.g., Chalmidia trachomatis; Neisseria, e.g. Neisseria meningitidis; and Legionella, e.g. Legionella pneumophilia; and also the obligately parasitic members of the order Rickettsiales.

Appropriate dosages of the macrolide will of course vary depending upon the condition to be treated (for example the severity of the overdose or the disease type), the effect desired, the mode of administration and the like.

In general however satisfactory results are obtained on administration orally at dosages of the order of from 0.05 to 10mg/kg/day, preferably 0.1 to 7.5 mg/kg/day, more preferably 0.1 to 2 mg/kg/day, administered once or, in divided doses, 2 to 4 times per day. On administration parenterally, for example by i.v. drip or infusion, dosages on the order of from 0.01 to 5 mg/kg/day, preferably 0.05 to 1.0 mg/kg/day and more preferably 0.1 to 1.0 mg/kg/day can be used.

Suitable daily dosages for patients are thus of the order of from 2.5 to 500 mg p.o., preferably 5 to 250 mg p.o., more preferably 5 to 100 mg p.o., or of the order of from 0.5 to 250 mg i.v., preferably 2.5 to 125 mg i.v. and more preferably 2.5 to 50 mg i.v..

Dosaging may also be arranged in a patient specific manner to provide pre-determined trough blood levels, as determined by the RIA technique. Thus patient dosaging may be adjusted to achieve regular on-going trough blood levels, as measured by RIA, of the order of from 50 to 1000 ng/ml, preferably 150 to 500 ng/ml; analogously to methods of dosaging currently employed for Ciclosporin immunosuppressive therapy.

The macrolide of formula I may be administered by any conventional route, in particular enterally or parenterally. Suitable enterally administered forms are solutions for drinking, tablets or capsules. Suitable parenteral forms are injectable solutions or suspensions. Suitable unit dosage forms for oral administration may comprise from 1 to 50 mg of the macrolide of formula I; usually 1 to 10 mg.

The macrolide of formula I may be produced synthetically, e.g. by total synthesis using a procedure analogous to that described for rapamycin by Nicolaou, et al., *J. Am. Chem. Soc.* (1993) **115**: 4419, or by fermentation as described below, or by a combination of synthetic and biosynthetic means, e.g., by isolating fermentation products and further chemically modifying them.

The macrolide of formula I is produced by a newly isolated Streptomyces strain, Streptomyces sp. A 91-261402 which was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-3300 Braunschweig, Germany, under the terms of the Budapest Treaty, on 3 December 1992 and is identified by Accession number DSM 7348.

The invention includes biologically pure isolates of the strain Streptomyces sp. A 91-261402 (DSM 7348) and mutants, recombinants and modified forms thereof which are capable of producing the macrolide of Formula I.

The isolation and characteristics of this new strain are described in greater detail in Example 1.

The macrolide of formula I may be obtained by cultivating the strain in an appropriate culture medium and then isolating the macrolide by chromatography.

Therefore, in another aspect, this invention provides a process for the production of a macrolide of formula I comprising cultivating Streptomyces sp. A 91-261402 (DSM 7348) or a mutant, recombinant or modified form thereof in an appropriate culture medium and isolating the macrolide.

Preferably the sources of carbon in the culture medium are carbohydrates such as glucose, xylose, galactose, glycerin, starch, and dextrin. Preferred sources of nitrogen are yeast extract, meat extract, peptone, gluten meal, cottonseed meal, soybean meal, casein hydrolysates, soybean hydrolysates, yeast hydrolysates, and the like and inorganic and organic nitrogen-containing compounds such as ammonium salts, urea, amino acids and the like.

Conventional fermentation agents and trace materials may also be added.

Preferably the fermentation is conducted under submerged aerobic conditions at a temperature between 20 and 40°C, more preferably between 23°C and 27°C.

The macrolide of formula I may also be used as a diagnostic tool to determine the presence of FK506 type immunosuppressants in broths. This can be done using standard competitive assays based on the FK506 antagonistic properties of the macrolide of formula I. Preferably the macrolide of formula I is immobilised in microtiter wells and then allowed to bind in the presence of a test broth to labelled macrophilin-12.

The invention is further described by way of example only with reference to Figures 1 and 2 in which;

Figure 1 is the infrared spectrum of the macrolide of formula I, and

Figure 2 is the proton NMR spectrum of the macrolide of formula I.

EXAMPLES:

Example 1: Description and Fermentation of strain Streptomyces sp. DSM 7348

Description of the strain

Streptomyces sp. A91-261402 was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-3300 Braunschweig, Germany on December 3 1992 under the terms of the Budapest Treaty, and has been assigned the reference number DSM 7348.

Streptomyces sp. A91-261402 was isolated from a soil sample (pH 7,7) collected along the Virgin River, Utah, U.S.A.

The strain belongs to the genus Streptomyces according to the description in Bergey's Manual, 8th edition, 1974, the new edition of the Bergey'Manual (1989) and The Prokaryotes (1992).

The cell walls contain LL-diaminopimelic acid. The fatty acids are iso- and anteiso-branched straight and unsaturated. The sugar spectrum is non distinctive. The vegetative mycelium does not break down in fragments. The aerial mycelium forms long chain of spores.

According to the previous cited reference books, the strain DSM 7348 is a new Streptomyces, designated A91-261402.

The strain DSM 7348 grows on various organic and inorganic media and in most cases form aerial mycelium. The primary substrate mycelium grows as hyphae and is generally beige. The aerial mycelium is white to white-greyish and forms long chains of spores which belong to the type "verticillus spira".

The ability of Streptomyces sp. A91-261402 to grow on usual biological media,

its carbon utilization, and its physiological characteristics are presented below following tables.

Growth on various biological media:

Culture medium	Culture characteristics
yeast extract/ malt agar	growth: good substrate mycelium: beige aerial mycelium: white soluble pigment: none
oatmeal	growth: good substrate mycelium: beige aerial mycelium: white-greyish soluble pigment: none
glucose-asparagine	growth: good substrate mycelium: spare, whitish soluble pigment: none
nutrient agar	growth: medium weak substrate mycelium: beige aerial mycelium: none soluble pigment: none
Inorganic salts/ starch agar	growth: good substrate mycelium: yellowish aerial mycelium: grey soluble pigment: none
Sucrose/ nitrate agar	growth: poor substrate mycelium: white-beige aerial mycelium: spare, white soluble pigment: none
Glycerol/ asparagine agar	growth: good substrate mycelium: beige aerial mycelium. white to grey

soluble pigment: none

carbon utilization

good: glucose, xylose, mannose, rhamnose, raffinose, m-inositol
poor: arabinose, sucrose, fructose
negative: cellulose

physiological characteristics

nitrate reduction: weak
starch hydrolysis: very weak
tyrosine degradation: positive
milk peptonisation: positive
melanin formation: negative
growth temperatures: 13-37°c. No growth at 45°C.
pH-range: 5-9
NaCl resistance: up to 6%. Reduced growth at 6%.

As with any microorganism, Streptomyces sp. A91-261402 can be mutated or modified into different forms by conventional technique, e.g., by UV radiation or by treatment with a chemical mutagen such as N-methyl-N'-nitro-nitrosoguanidine. Recombinant clones can be obtained by protoplast fusion. All such mutants or recombinants or modified forms, capable of producing the macrolide 222-662 in a quantity greater than 10 mg/l of culture fall within the scope of this application.

Culture conditions

The new strain DSM 7348 may be cultured at suitable temperatures on various culture media using appropriate nutrients and mineral substances, as aerobic or immersion cultures. The fermentation media should contain a utilisable source of carbon, sources of nitrogen and mineral salts including

trace elements, all of which can be added in the form of well defined products or as complex mixtures, as are found in biological products of various origins. The following fermentation description describes the original conditions under which the macrolide of Formula I was discovered. Improvements of the yield can be achieved by optimisation of the culture conditions (aeration, temperature, pH, quality and quantity of the carbon and nitrogen sources, quantity of the mineral salts and of the trace elements) and by controlling the fermentation conditions in bioreactors.

Fermentation

Culture of strain DSM 7348

a. Agar starting culture

Agar slant cultures of the strain DSM 7348 are grown for 10 to 14 days at 27°C on the following agar medium:

Glucose	10.0g
Soluble starch	20.0g
Yeast extract (Gistex, Gist Brocades)	5.0g
NZ-Amine,Type A (Sheffield)	5.0g
Calcium carbonate	1.0g
Agar (Bacto)	15.0g
Demineralised water to 1000ml	

The medium is adjusted to pH 6.6-6.8 with $NaOH/H_2SO_4$, then sterilised for 20 min. at 120°C.

The cultures can be stored at -25°-70°C. A suspension in glycerol-peptone can be stored under liquid nitrogen.

b Preculture

Spores and mycelium of one starting culture are suspended in 10 ml of a 0.9% salt solution. Three 200ml Erlenmeyer flasks each containing 50ml of preculture medium are inoculated with 2.5ml of this suspension. The composition of the preculture medium is as follows:

Cerelose (COST)	7.5g
L-asparagine	1.0g
Yeast extract (BBL)	1.35g
Glycerin	7.5g
Malt extract liquid (Wander)	7.5g
Starch soluble	7.5g
Soya protein (Siegfried)	7.5g
NZ-Amine,Type A (Sheffield)	2.5g
$CaCO_3$	0.050g
KH_2PO_4	0.250g
K_2HPO_4	0.500g
$MgSO_4.7H_2O$	0.100g
NaCl	0.050g
Trace element solution A	1ml
Agar (Bacto)	1g
Demineralised water to 1000ml	

The medium is adjusted to pH 6.8-7.2 with $NaOH/H_2SO_4$ and sterilised for 20m at 121°5C.

The composition of the trace element solution A is as follows:

$FeSO_4.7H_2O$	5.0g
$ZnSO_4.7H_2O$	4.0g
$MnCl_2.4H_2O$	2.0g
$CuSO_4.5H_2O$	0.2g
$CoCl_2.6H_2O$	2.0g

H_3BO_3	0.1g
KI	0.05g
H_2SO_4 (95%)	1 ml
Demineralised water to	1000ml

c. Intermediate culture

The preculture is fermented for 96 hr. at 27°C on a rotary shaker at 200 rpm with an eccentricity of 50 mm.

12 200ml Erlenmeyer flasks, each containing 50ml of the preculture medium, are inoculated with 5ml of the preculture and fermented for 72 hr. at 27°C on a rotary shaker at 200 rpm with an eccentricity of 50mm.

d. Main culture

Fifty 500ml Erlenmeyer flasks, each containing 100ml of the main medium, are each inoculated with 10ml of the intermediate culture. Fermentation is carried out at 24°C for 6 days on a rotary shaker at 200 rpm with an eccentricity of 50mm. The composition of the main culture medium is as follows:

Cerelose (COST)	20g
Malt extract liquid (Wander)	2g
Yeast extract (Bacto)	2g
Soytone (Bacto)	2g
KH_2PO_4	0.2g
K_2HPO_4	0.4g
$MgSO_4.7H_2O$	0.2g
NaCl	0.05g
$CaCl_2.6H_20$	0.05g

Trace element solution A	1ml
Agar (Bacto)	1g

demineralised water to 1000ml.

The pH is adjusted to 6.3 with KOH/HCl. The medium is sterilised for 20 min at 121°C.

Example 2: Isolation of the macrolide of formula I

5 l of preculture fermentation broth obtained as in Example 1 part (b) is filtered through a thick paper filter to separate off the mycelia. The mycelia are treated with methanol, under vigorous stirring, for about 15 minutes and filtered. The filtrate is then concentrated and combined with the first filtrate and the resulting mixture is extracted twice with 5 l ethyl acetate and twice with 5 l n-butanol. The two extracts are evaporated to dryness on a rotary evaporator under reduced pressure. The two residues obtained (1.4 g from the ethyl acetate extraction and 6.5 g from the n-butanol extraction), both active in a macrophilin binding assay, are combined and subjected to chromatography on Sephadex LH20 in methanol solution. Eight fractions are collected. The fractions are subjected to macrophilin binding assay analysis and active fractions 3 and 4 (2.7 g) are further separated on a column of 3 kg Lichroprep RP18 40-64 µm using methanol/water in the ratio 8:2. The fractions are then subjected to macrophilin binding assay analysis and active fractions 5 to 7 are then purified on a column of 15 g silica gel SICAM using methylene chloride/methanol/ water in the ratio 92:7.5:0.5. The fractions are again subjected to a macrophilin binding assay analysis and active fractions 3 and 4 are combined, filtered and evaporated to dryness under vacuum to yield 27 mg of pure amorphous macrolide.

Analysis of the macrolide of formula I gives the following data:

Fp. 112-114°C (amorphous), molecular formula $C_{45}H_{75}NO_{12}$(822.1)

Opt.rotation: $[\alpha]_D^{20} = -1.0^0$ (c= 0.91 MeOH)

UV (MeOH): Endabsorption

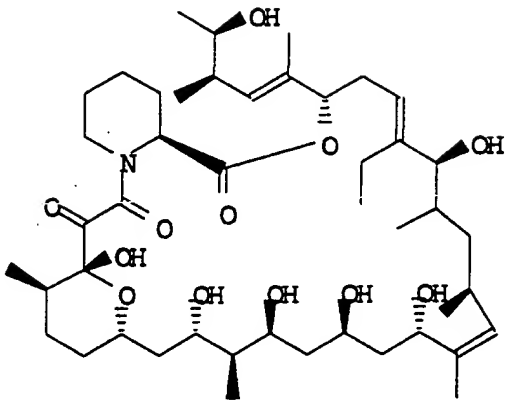
IR (KBr crystal) the spectrum given in Figure 1

Mass spectra peaks at (m/e) 324, 336, 364, 390, 750, 768, 786, 804, 822(MH^+), 844(M^+Na^+).

NMR (^{1}H-nmr, 500 HZ, DMSO), the spectrum is given in Figure 2

CLAIMS

1. A macrolide of the formula I



I

2. A biologically pure isolate of strain Streptomyces sp. A 91-261 402 (DSM 7348) and mutants, recombinants and modified forms thereof which are capable of producing the macrolide of Formula I.

3. A process for the production of a macrolide of formula I comprising cultivating the strain Streptomyces sp. A 91-261 402 deposited under the number DSM 7348 or a mutant, recombinant or modified form thereof in an appropriate culture medium and isolating the macrolide by chromatography.

4. A macrolide when produced by a process of claim 3.

5. A macrolide according to claim 1 or claim 4 for use as a pharmaceutical.

6. A pharmaceutical composition comprising a macrolide according to claim 1 or claim 4 together with a pharmaceutically acceptable diluent or carrier.

7. Use of a macrolide according to claim 1 or claim in the manufacture of a medicament for use as:

(i) an antidote for an overdose of a macrophilin binding immunosuppressant FK506 or rapamycin,
(ii) a steroid potentiator, or
(iii) an anti-infective agent for infections or infectious diseases caused by organisms producing Mip or Mip-like factors.

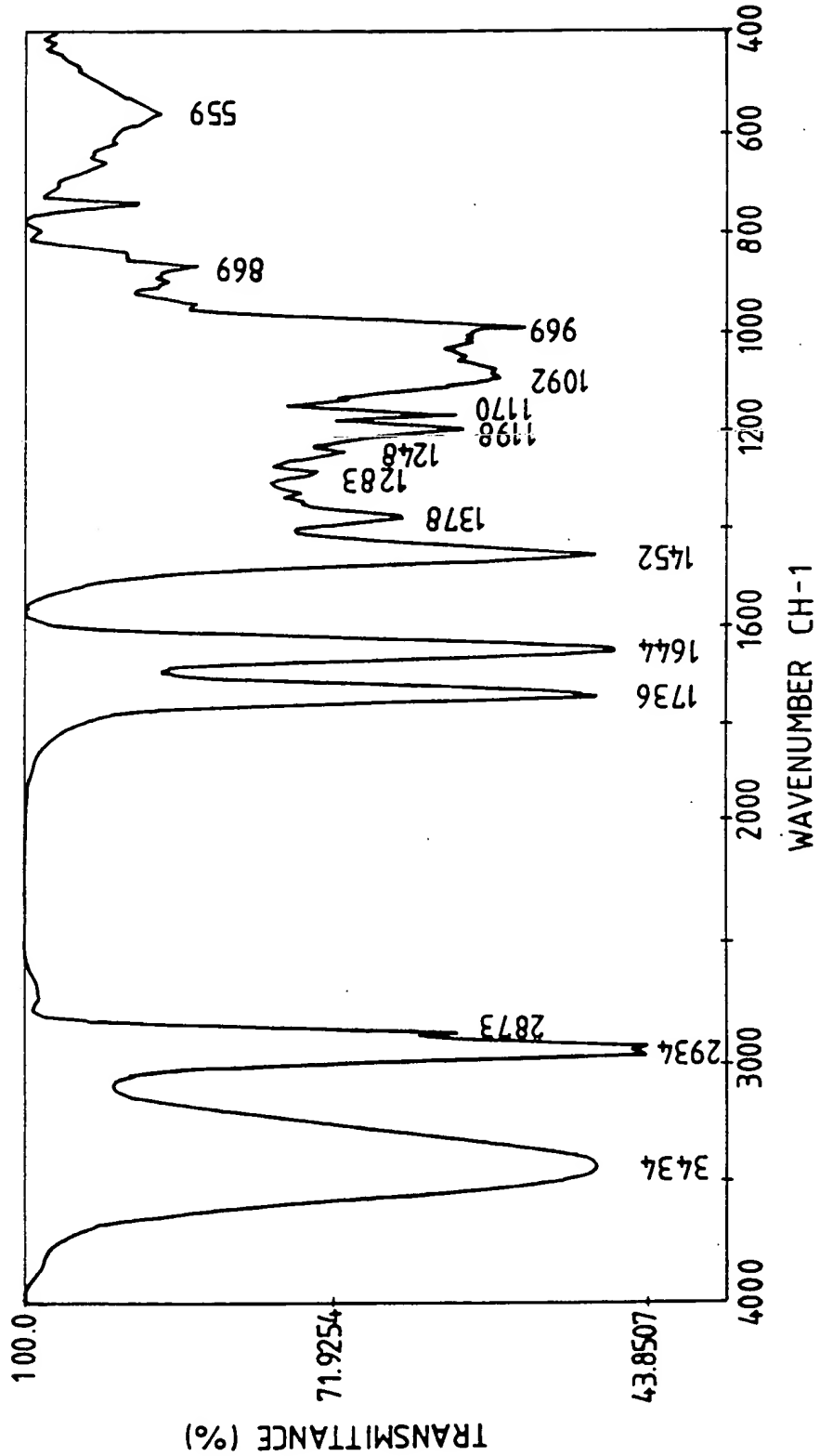
8. A combination of a macrolide according to claim 1 or 4 and a glucocorticosteroid for use in the treatment of inflammatory/hyperproliferative skin diseases.

9. A pharmaceutical composition comprising the combination of a macrolide according to claim 1 or 4 and a glucocorticosteroid, for use in the treatment of inflammatory/hyperproliferative skin diseases.

10. Use of the combination according to claim 8 for the preparation of a medicament for the treatment of inflammatory/hyperproliferative skin diseases.

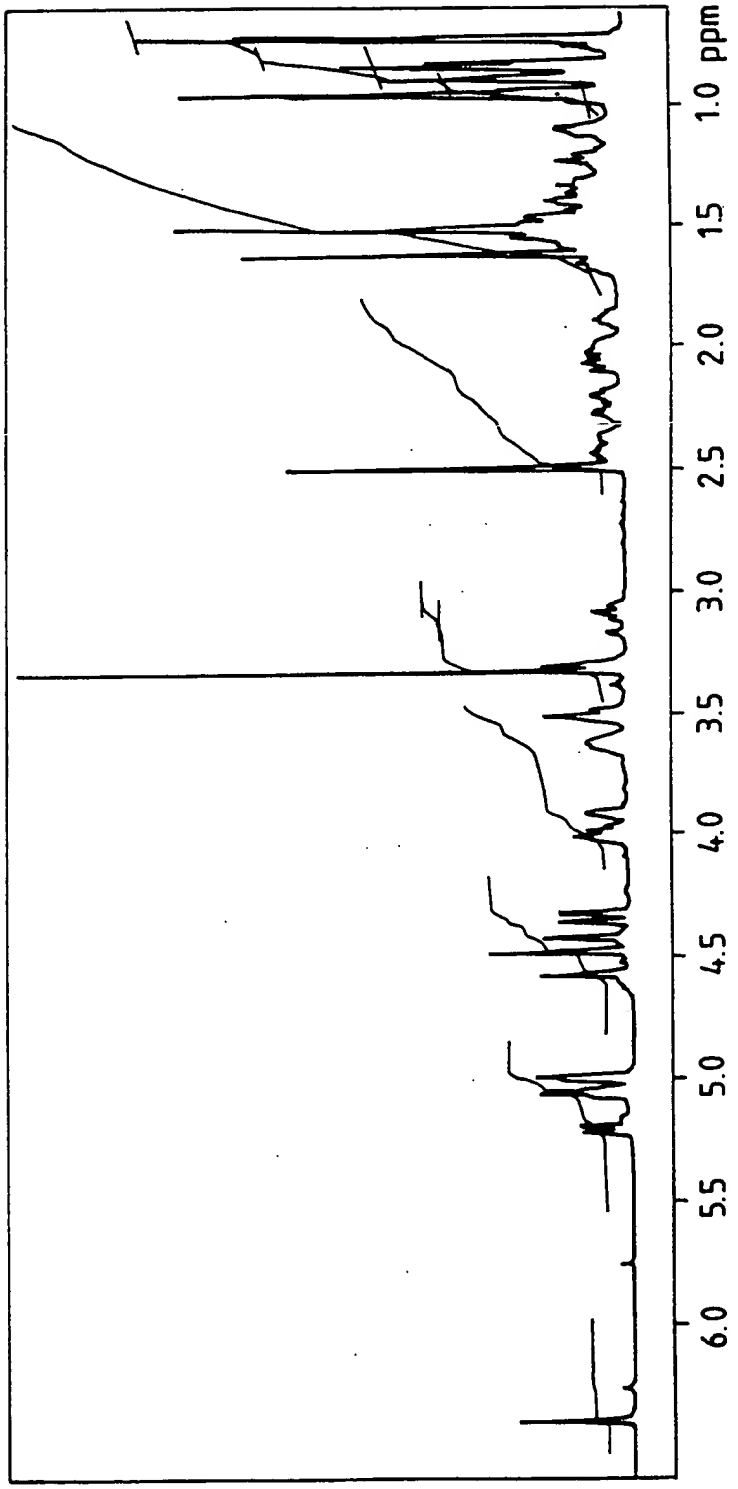
11. A method of treatment for inflammatory/hyperproliferative skin diseases which comprises administering a therapeutically effective amount of a combination according to claim 8 to a subject in need of such treatment.

FIG:1



SUBSTITUTE SHEET

FIG. 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 94/00284

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07D498/18 C12P17/18 A61K31/435 //(C07D498/18,311:00, 273:00,221:00),(C12P17/18,C12R1:465)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07D C12P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE vol. 250, no. 4980 , 26 October 1990 , LANCASTER, PA US pages 556 - 559 B. E. BIERER ET AL 'Probing immunosuppressant action with a nonnatural immunoplilin ligand' see the whole document -----	1

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
11 May 1994	24. 05. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Voyiazoglou, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 94/00284

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 11 is directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.